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CAMAG LABORATORY

Method ID	Identification of Echinacea	Author	Date
MOA 004		AS	24.Oct.2005

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Validated Method

1. Purpose of method

The method for identification of roots of Echinacea species by HPTLC fingerprint is suitable to identify a given sample of plant material as Echinacea root of one of the species *Echinacea purpurea, E. angustifolia,* or *E. pallida* based on its alkylamide and phenylpropanoid fingerprint. Each of the three species yields characteristic fingerprints, which allow identification with certainty.

Adulterants, such as *Echinacea atrorubens, Liatris punctata,* and *Parthenium integrifolium* show a different profile.

2. Materials

Wear lab coat, protective goggles and gloves at all times when handling chemicals.

2.1 Chemicals and solvents

Acetic acid, anisaldehyde, cyclohexane, dichloromethane, diphenylborinic acid aminoethylester, ethyl acetate, ethylmethyl ketone, formic acid 98-100%, methanol, sulfuric acid, toluene, all of “for analysis” or HPLC quality, distilled or demineralized water.

2.2 Samples and reference materials (optional)

Botanically authenticated and freshly dried *Echinacea purpurea, E. angustifolia* and *E. pallida* root.
2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide, β-sitosterol, echinacoside, cynarin, chlorogenic acid, caffeic acid, caftaric acid, and cichoric acid [ChromaDex].

2.3 Plates

Glass plates HPTLC Si 60 F_{254}, 10x10 or 20x10 cm, Merck (Darmstadt, Germany), or others if equivalence was shown.

2.4 Lab ware and instruments

- Analytical mill or mortar,
- ultrasonic bath,
- centrifuge with centrifuge tubes, or suitable set-up for filtration with beakers or small flasks (10 or 20 mL)
- analytical balance,
- graduated pipettes (1, 5, and 10 mL),
- graduated cylinder (50 mL),
- glass bottles (with tightly closing lid, 100 mL and 200 mL),
- TLC Twin Trough Chamber or Flat Bottom Chamber 20x10 cm, alternatively automatic developing chamber,
- sample application device using the spray-on technique (such as Linomat, ATS [CAMAG] or AS 30 [Desaga]),
- chromatogram immersion device [CAMAG],
- plate heater or oven,
- documentation system consisting of an illumination device for UV 254 nm, UV 366 nm, and white light and a video or digital camera,
- suitable TLC software,
- thermometer and hygrometer
- lab coat, protective goggles and gloves.

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3a. Description of method for the alkylamide profile

3.1a Preparation of test solutions

3.1.1a Raw materials

Mill each sample to a fine powder. Weigh 1 g each of powder in individual centrifuge tubes or flasks. Add 10 mL of dichloromethane each and mix well. Sonicate for 10 min. Centrifuge or filter the solutions and use the supernatants / filtrates as test solutions.

3.2a Preparation of reference solutions (optional)

3.2.1a Botanical reference solution

As 3.1.1

3.2.2a Chemical reference solutions

Weigh 1 mg of 2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide in a flask. Add 5 mL of methanol. Dissolve β-sitosterol in the same way.

3.3a Preparation of derivatizing reagent

Place 170 mL of methanol in a 200 mL glass bottle and cool it down in a water-ice cubes-salt bath or in a freezer. To the ice-cold methanol add slowly and carefully 20 mL of acetic acid and 10 mL of sulfuric acid and mix well. Allow the mixture to cool to room temperature, then add 1 mL of anisaldehyde.

3.4a Stationary phase

10x10 cm (or 20x10 cm) glass plates HPTLC silica gel 60 F_{254} (Merck).

3.5a Sample application

Apply 5 µL of test solution, 5 µL of botanical reference solution, and 2 µL of each chemical reference solution each as 8 mm band, at least 2 mm apart, 8 mm from the lower edge and at least 15 mm from left and right edges of the plate.

3.6a Temperature and humidity

Record temperature and humidity in the laboratory.

3.7a Chromatography

3.7.1a Developing solvent

Place 80 mL of toluene, 20 mL of ethyl acetate, 10 mL of cyclohexane, and 3 mL of formic acid in a bottle, close lid tightly and mix content by shaking. Larger or smaller amounts of solvent can be prepared once a day.

3.7.2a Chamber

Line one side of a 10x10 cm Twin Trough Chamber with filter paper. Pour 10 mL of developing solvent over the paper, and tilt the chamber to equilibrate solvent level in both troughs, close the lid. Allow the chamber to saturate for 20 min. If using a 20x10 cm chamber, use 20 mL of developing solvent. If using a Flat Bottom Chamber, use enough solvent to cover the bottom with a 5 mm level. If using an automatic chamber, refer to the manufacturer's instructions.

3.7.3a Development

Measure and mark on the plate the developing distance of 70 mm from lower edge of plate (62 mm from application position). Open the saturated chamber and introduce the plate with the layer facing the inside, close the chamber and wait for the solvent to reach the mark. Remove the plate from the chamber.

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3.7.4a Drying

Dry the plate for 5 min with cold air (hair dryer).

3.8a Documentation and derivatization

3.8.1a Documentation of non-derivatized plate

No documentation is needed.

3.8.2a Derivatization

Turn on plate heater or oven and select temperature (100°C). Charge the tank of the immersion device with 200 mL of reagent. Place plate in holder of immersion device, set parameters (speed:5, time:0) and press start. Let excess reagent drip off the plate, wipe off the back of the plate with a paper towel. Remove plate from plate holder. Place plate onto plate heater or in oven. Remove hot plate after 3-5 min and let it cool down to room temperature.

3.8.3a Documentation of derivatized plate

Document the plate using illumination with white light (reflection and transmission) within 4 min after the completion of derivatization.

3.9a Results

Compare the images of the plate obtained under 3.8.3a with the image provided under 4.1a. The plate can only be evaluated if it passes the system suitability test (4.3a).
Evaluate the results obtained with the test solution according to the description under 4.2a. The test solution can be identified as one of the three *Echinacea* species if the fingerprint obtained is similar to that of the corresponding BRM. In comparison to the BRM, the test solution doesn't show any additional intense zone after derivatization. None of the described zone is missing.

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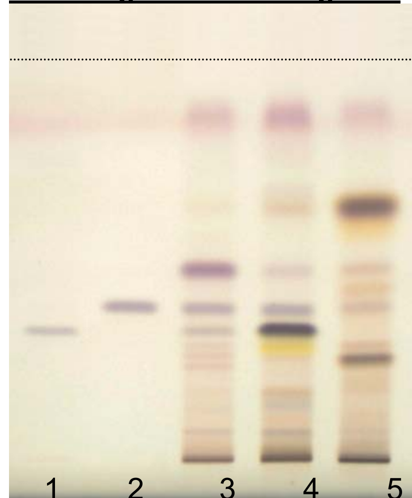
4a. Results for comparison: Alkylamide profile

4.1a Image of chromatograms



Track assignment

1: 2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide
2: β-sitosterol
3: *E. purpurea root*
4: *E. angustifolia root*
5: *E. pallida root*

4.2a Description of results

The chemical reference standards 2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide (DAI) and β-sitosterol show a blue violet zone at R_F=0.31 and R_F=0.38 respectively. BRMs of each species show a prominent violet zone corresponding to in color and position to β-sitosterol and another violet zone at R_F=0.47 which is particularly strong for *E. purpurea root* and week for *E. angustifolia root* and *E. pallida root*. Close to the solvent front a broad pink violet zone is seen.

- *E. purpurea root* and *E. angustifolia root* must show a prominent zone corresponding in color and position to DAI. This zone is absent in *E. pallida root*.
- *E. angustifolia root* shows a characteristic yellow zone below DAI. (**NOTE:** This zone turns reddish pink when the plate is heated at 100°C for more than 10 min!)
- *E. pallida root* shows two brownish zones below the position of DAI and a characteristic broad olive green/yellow double zone at R_F=0.60. *E. purpurea root* and *E. angustifolia root* show only faint zones at this position.

4.3a System suitability test

The result obtained in the test is suitable for evaluation if the following requirements are met:
E. purpurea root: The zone corresponding to β-sitosterol is clearly separated from the zones above and below.
E. angustifolia root: the zone of DAI and the yellow zone directly below are clearly separated.
E. pallida root: The zone corresponding to β-sitosterol is clearly separated.

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3b. Description of method for the phenylpropanoid profile

3.1b Preparation of test solutions

3.1.1b Raw materials

Mill each sample to a fine powder. Weigh 1 g each of powder in individual centrifuge tubes or flasks. Add 10 mL of methanol each and mix well. Sonicate for 10 min. Centrifuge or filter the solutions and use the supernatants / filtrates as test solutions.

3.1.2b Dry extracts and dry finished products

Weigh an amount of each extract powder or finished product equivalent to 1 g of raw material in individual centrifuge tubes or flasks. Add 10 mL of methanol each and mix well. Sonicate for 10 min. Centrifuge or filter the solutions and use the supernatants / filtrates are as test solutions.

3.1.3b Liquid extracts and liquid finished products

Dilute the liquid samples with the same solvent (as on the label) to obtain a solution with the same concentration as that of a test solution from raw material as described under 3.1.1.

3.2b Preparation of reference solutions (optional)

3.2.1b Botanical reference solution

As 3.1.1

3.2.2b Chemical reference solutions

Weigh individually 2 mg of echinacoside, 2 mg of cynarin, 1 mg of chlorogenic acid, 1 mg of caffeic acid, 0.5 mg of caftaric acid, and 0.5 mg of cichoric acid into suitable vials. Add 10 mL of methanol to each vial and dissolve by mixing.

3.3b Preparation of derivatizing reagent

Dissolve 1 g of diphenylborinic acid aminoethylester in 200 mL of ethyl acetate.

3.4b Stationary phase

10x10 cm (or 20x10 cm) glass plates HPTLC silica gel 60 F_{254} (Merck).

3.5b Sample application

Apply 5 µL of test solution, 5 µL of botanical reference solution, and 2 µL of each chemical reference solution each as 8 mm band, at least 2 mm apart, 8 mm from the lower edge and at least 15 mm from left and right edges of the plate.

3.6b Temperature and humidity

Record temperature and humidity in the laboratory.

3.7b Chromatography

3.7.1b Developing solvent

Place 50 mL of ethyl acetate, 30 mL of ethylmethyl ketone, 10 mL of water, and 10 mL of formic acid in a bottle, close lid tightly and mix content by shaking. Larger or smaller amounts of solvent can be prepared once a day.

3.7.2b Chamber

Line one side of a 10x10 cm Twin Trough Chamber with filter paper. Pour 10 mL of developing solvent over the paper, and tilt the chamber to equilibrate solvent level in both troughs, close the lid. Allow the chamber to saturate for 20 min. If using a 20x10 cm chamber, use 20 mL of developing solvent. If using a Flat Bottom Chamber, use enough

solvent to cover the bottom with a 5 mm level. If using an automatic chamber, refer to the manufacturer's instructions.

3.7.3b Development
Measure and mark on the plate the developing distance of 70 mm from lower edge of plate (62 mm from application position). Open the saturated chamber and introduce the plate with the layer facing the inside, close the chamber and wait for the solvent to reach the mark. Remove the plate from the chamber.

3.7.4b Drying
Dry the plate for 5 min with cold air (hair dryer).

3.8b Documentation and derivatization
3.8.1b Documentation of non-derivatized plate
No documentation is needed.

3.8.2b Derivatization
Turn on plate heater or oven and select temperature (100°C). Charge the tank of the immersion device with 200 mL of reagent. Heat the plate at 100°C for 5 min. Place plate while still warm in holder of immersion device, set parameters (spped:5, time:0) and press start. Let excess reagent drip off the plate, wipe off the back of the plate with a paper towel. Remove plate from plate holder and leave it to dry for 5 min in the fume hood.

3.8.3b Documentation of derivatized plate
Document the plate using illumination with UV366 nm.

3.9b Results
Compare the images of the plate obtained under 3.8.3b with the image provided under 4.1b. The plate can only be evaluated if it passes the system suitability test (4.3b).
Evaluate the results obtained with the test solution according to the description under 4.2b. The test solution can be identified as one of the three *Echinacea* species if the fingerprint obtained is similar to that of the corresponding BRM. In comparison to the BRM, the test solution doesn't show any additional intense zone after derivatization. None of the described zone is missing.

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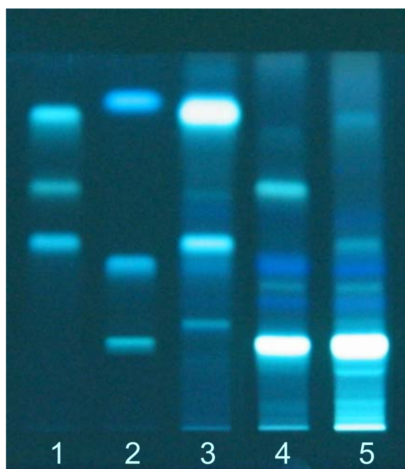
4b. Results for comparison: Phenylpropanoid profile

4.1b Image of chromatograms



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Track assignment

1: Caftaric acid, cynarin, cichoric acid (with increasing R_F)
2: Echinacoside, chlorogenic acid, caffeic acid (with increasing R_F)
3: *E. purpurea root*
4: *E. angustifolia root*
5: *E. pallida root*

4.2b Description of results

The chemical reference substances give blue-white fluorescing zones in the following order (increasing R_F): Echinacoside (0.22), chlorogenic acid (0.44), caftaric acid (0.50), cynarin (0.64), cichoric acid (0.84), caffeic acid (0.87).

- *E. purpurea root* shows a prominent zone corresponding to caftaric acid and a very strong zone corresponding to cichoric acid. Other weak zones may be present but **NO** zones are seen at the positions of echinacoside and cynarin.
- *E. angustifolia root* shows a very strong zone corresponding to echinacoside and a prominent zone corresponding to cynarin. A weak zone at or slightly below the position of cichoric acid can be present. Several weak zones may be present between the positions of echinacoside and cynarin but **NO** zone is seen at the position of caftaric acid.
- *E. pallida root* shows a very strong zone corresponding to echinacoside. Weak zones corresponding to caftaric acid and cichoric acid are present. Several weak zones are seen above and below the position of echinacoside but **NO** zone is seen at the position of cynarin.

4.3b System suitability test

The result obtained in the test is suitable for evaluation if the following requirements are met:
E. purpurea root: the position of the zone corresponding caftaric acid must be at R_F=0.50.
E. angustifolia root and *E. pallida root:* the position of the zone corresponding echinacoside must be at R_F=0.22. R_F values may vary by +/- 0.05.
Optional: The chemical reference standards chlorogenic acid and caftaric acid are clearly separated.

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5. Approvals

Validation approved:
Date: 12. August 2005, **by:** ER

MOA 004 released:
Date: , **by:** , **Signature:**

Revision history

Creation date
24. October 2005/AS

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